For general laboratory use.
FOR *IN VITRO* USE ONLY.

Pwo DNA Polymerase

From Pyrococcus woesei
Deoxynucleoside-triphospate:
DNA deoxynucleotidyltransferase, EC 2.7.7.7

Cat. No. 1 644 947 100 units
Cat. No. 1 644 955 500 units (2 × 250 units)

Version 3, Nov. 1999
Store at −15 to −25° C

Product description

Volume activity
1–5 x 10^3 units/ml as determined in the assay on activated DNA.

Storage and dilution buffer
20 mM Tris-HCl, pH 7.5 (20° C), 100 mM KCl, 1mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Nonidet[1] P40 (v/v), 0.5% Tween[2] 20 (v/v), 50% glycerol (v/v).

Stability
The undiluted enzyme solution is stable when stored at −15 to −25° C at least until the date stated on the label.

Supplied buffers and solution
- **PCR buffer, 10 x conc. with $MgSO_4$:** 100 mM Tris-HCl, pH 8.85 (20° C), 250 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$
- **PCR buffer, 10 x conc. without $MgSO_4$:** 100 mM Tris-HCl, pH 8.85 (20° C), 250 mM KCl, 50 mM $(NH_4)_2SO_4$
- **$MgSO_4$ stock solution:** 25 mM $MgSO_4$

Unit assay on activated DNA
a) Incubation buffer for assay on activated DNA: 20 mM Tris-HCl, pH 8.8 (20° C), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.2 mM of each dATP, dCTP, dGTP, dTTP.

b) Incubation procedure: 12.5 mg activated (1) calf thymus DNA* and 0.1 mCi [α-^{32}P]dCTP are incubated with 0.01–0.1 units Pwo DNA polymerase in 50 µl incubation buffer with a paraffin-oil overlay at 70° C for 30 min. The amount of incorporated dNTPs is determined by trichloroacetic acid precipitation followed by scintillation counting.

Unit definition
One unit Pwo DNA polymerase is defined as the amount of enzyme that catalyzes the incorporation of 10 nmol total deoxynucleoside triphosphates into acid precipitable DNA within 30 min at 70° C under the conditions described above.

Properties
Pwo DNA polymerase was originally isolated from the hyperthermophilic archaebacterium Pyrococcus woesei. The enzyme has a molecular weight of about 90 kD. It is a highly processive 5'–3' DNA polymerase and possesses an 3'–5' exonuclease activity also known as proofreading activity. The enzyme has no detectable 5'–3' exonuclease activity.

Pwo DNA polymerase exhibits increased thermal stability with a half life of greater than 2 h at 100° C compared to Taq DNA polymerase with a half life of less than 5 min at this temperature.

The inherent 3'–5' exonuclease proofreading activity of Pwo results in an over 10-fold increased fidelity of DNA synthesis compared to Taq DNA polymerase. Pwo DNA polymerase generated PCR products are blunt-ended and can therefore be used directly for blunt-end ligation without any pretreatment of the ends.

Fidelity of *in vitro* DNA polymerization is one of the most important subjects in PCR. For many applications of PCR, where a homogenous DNA population is analyzed (i.e. direct sequencing or restriction endonuclease digestion), the mutations that are induced by the polymerase during PCR are of little concern. However if only a small amount of template DNA or RNA is used as starting material and if after PCR single DNA molecules are analyzed, PCR artifacts can be a significant problem.

Fidelity of DNA polymerization is for instance important for:

- cloning of PCR products
- study of allelic polymorphism in individual RNA transcripts (1, 2)
- characterisation of the allelic stage of single cells (3) or single DNA molecules (4, 5)
- characterisation of rare mutations in tissue (6)
- characterisation of a population of cells in culture

When using Taq DNA polymerase [error rate of 2 x 10^{-4} errors/base (7)] about 56% of a 200 bp amplificaton product will contain at least a single error after 1 million fold amplification. In contrast when using Pwo DNA polymerase for amplification only 10% of the products will contain an error under the same conditions.

Application protocols

The optimal reaction conditions (incubation times and temperatures, concentration of Pwo DNA polymerase, template DNA, Mg^{2+}-ions) depend on the template/primer pair and must be determined individually. It is especially important to titrate the Mg^{2+}-concentration and the amount of enzyme required per assay. Optimal Mg^{2+} concentrations are in the range of 1–10 mM.

Whereas Taq DNA polymerase requires $MgCl_2$ for optimal activity Pwo shows higher activity with $MgSO_4$. The standard concentration of Mg^{2+} is 2.0 mM for Pwo. Optimal enzyme concentrations range from 0.5–5 units per assay. The standard concentration is 2.5 units.

The dNTPs (e.g. PCR Nucleotide Mix, Cat. No. 1581 295) should be added to the incubation mixture directly before use. This will prevent decomposition of deoxynucleoside triphosphates that can occur at the alkaline pH required for optimal enzyme activity.

Note
In the absence of dNTPs, the 3'–5' exonuclease activity associated with Pwo will begin to degrade template and primer DNA. Therefore, it is important to always add Pwo DNA polymerase to the reaction mixture last. This can be achieved by using the hot start technique with AmpliWax[3]. In this case primer and template are separated from the polymerase by preparing appropriate upper and lower mixes. Both mixes are separated by a wax layer, that melts above 70° C and allows then thorough mixing of the reagents.

General protocol for DNA amplification

We recommend to prepare two master mix as follows:

1. Set up in a sterile microfuge tube on ice:

- for standard PCR set up a total PCR volume of 100 µl.
- for special amplifications e.g. circular plasmids GC rich templates or low amount of template but high yield of product desired set up a total PCR volume of 50 µl

Component	100 µl Volume	50 µl Volume	Final concentration
master mix 1:			
add sterile redist. H_2O	up to 50 µl	up to 25 µl	
dATP, 10 mM**	2 µl	1 µl	200 µM
dCTP, 10 mM**	2 µl	1 µl	200 µM
dGTP, 10 mM**	2 µl	1 µl	200 µM
dTTP, 10 mM**	2 µl	1 µl	200 µM
downstream primer	x µl	x µl	300 nM (100 µl) 600 nM (50 µl)
upstream primer	x µl	x µl	300 nM (100 µl) 600 nM (50 µl)
template DNA (e.g. human genomic DNA)	x µl	x µl	(0.1–0.75 µg)
master mix 2:			
add sterile redist. H_2O	up to 50 µl	up to 25 µl	
10 × PCR buffer with 20 mM $MgSO_4$	10 µl	5 µl	
Pwo DNA polymerase (5 U/µl)	0.5 µl	0.5 µl	2.5 U

Note

Instead of using single dNTP solutions the PCR Nucleotide Mix (Cat. No. 1 581 592) can be used.

- The preparation of 2 separate master mixes helps to circumvent the need of hot start and in addition avoids that the enzyme interacts with primers or template without dNTPs which could lead to a partial degradation of primer and template through the 3´–5´ exonuclease activity of Pwo.
- Alternatively use AmpliWax between mix 1 and mix 2. Only for 100 µl total PCR volume recommended, for 50 µl total PCR volume do not use AmpliWax.

2. Pipet together on ice master mix 1 and master mix 2 in a PCR tube (cat. nos. 1667 041, 1667 050), mix and overlay with 30 µl mineral oil if necessary (depending on the cycler used, when using 50 µl reaction volume an oil overlay is always recommended).
3. **Start cycling immediately.** An example for a cycle profile is given for the Perkin Elmer GenAmp 9600 Thermocycler. When using other Thermocyclers the cycle conditions have to be adjusted.

Note

When changing from Taq to Pwo in some cases problems have been observed for the amplification of the same target sequence. We recommend in these case to lower the annealing temperature by 2–3° C since the 3´–5´exonuclease activity of Pwo will shorten the primer during cycling.

1 ×	denature template 2 min at 94° C
10 ×	denaturation at 94° C for 15 s annealing usually at 45–65° C[1] for 30 s elongation at 72° C (45 s–2 min)
15–20 ×	denaturation at 94° C for 15 s annealing usually at 45–65° C[1] for 30 s elongation at 72° C (45 s–2 min)[2] + cycle elongation of 5 s for each cycle (e.g. cycle no. 11 has in addition 5 s cycle no. 12 has in addition 10 s cycle no. 13 has in addition 15 s etc.)
1 ×	use a proplonged elongation time up to 7 min at 72° C

1) annealing temperature depends on the melting temperature of the primer used.

2) We recommend elongation times as stated below (be aware that you should use cycle extension features):

Elongation time	45 s	1 min	2 min
PCR fragment lenght (kb)	up to 1	1.5	3

4. Analyze samples on a o.6%–1% agarose MP gel (Cat. Nos. 1 444 964, 1 388 983 or 1 388 991).

Note

Lack of an amplification product might be due to non optimal $MgSO_4$-concentration: In this case add in mix 2 10 µl PCR buffer, 10 × without $MgSO_4$ and add in addition:
(for 100 µl total volume)

25 mM $MgSO_4$ [µl]	6	8	10	12	14	16
Final Mg^{2+}concentration [mM]	1.5	2	2.5	3	3.5	4

Proceed as indicated in the general PCR protocol.

General notes

Magnesium concentration

$MgSO_4$ is preferred to $MgCl_2$. The standard concentration is 2 mM. The Mg^{2+} concentration should be optimized if little or no PCR product is obtained. The effect of magnesium on PCR efficiency is particularly pronounced for PCR products larger than 2 kB.

Detergents and other additives

Usually detergents will not improve PCR performance. Nevertheless in some cases improvements can be achieved by using up to 100 µg/µl BSA and/or 0.1% Triton[4] X100.

dNTP concentration

The nucleotide concentration should be at least 200 µM for each dNTP. Lower nucleotide concentrations might increase fidelity but can also activate 3'–5' exonuclease proofreading activity that might degrade primers and products.

Primer design

The 3'–5' exonuclease activity of Pwo DNA polymerase acts also on single stranded DNA (e.g. primer) in the absence and presence of dNTP. This activity does usuallly not interact with PCR performance. But it can be taken into consideration for primer design. To overcome slow degradation of primers nuclease resistant dNTPs can be used for primer synthesis. Additionally longer primers with maximized GC content and focussed complementarity at the 5'-end can be advantegeous.

Labeling with modified nucleotides

Pwo DNA polymerase accepts modified nucleotides like DIG-dUTP, Biotin-dUTP and Fluorescein-dUTP. The concentration of these nucleotides should be 50 µM (50 µM modified dUTP, 150 µM dTTP) for generating probes for Southern analysis. For Biotin-dUTP the magnesium concentration should be increased to 4 mM $MgSO_4$. For ELISA based detection systems a concentration of 10 µM modified dUTP is normally sufficient.

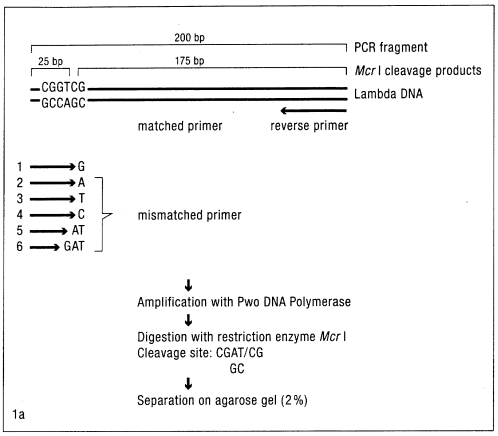
Carry over prevention with dUTP/UNG

dUTP alone instead of dTTP is a bad substrate for Pwo DNA polymerase. Therefore we do not recommend Pwo DNA polymerase in combination with UNG carry over prevention.

Cloning

Pwo DNA polymerase generated PCR products can be used directly for blunt-end ligation without prior filling in the ends with Klenow.

3'-mismatched primer correction assay



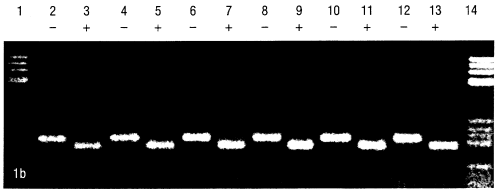


Fig 1:

a: Flowchart for 3' mismatched and matched primers: Mcr I recognizes CGPuPyCG.

b: PCR products of a 200 bp target from lambda DNA using perfectly matched and partially mismatched primers and Pwo DNA polymerase.

lane 1,14: DNA molecular weight marker V

lane 2,3: Primer I (G:C match)

lane 4,5: Primer II (G:A mismatch)

lane 6,7: Primer III (G:T mismatch)

lane 8,9: Primer IV (G:G mismatch)

lane 10,11: Primer V (2 base pair mismatch)

lane 12,13: Primer VI (3 base pair mismatch)

lane 2,4,6,8,10,12 (-): without restriction enzyme digestion (200 bp fragment)

lane 3,5,7, 9,11,13,(+): restriction enzyme digestion with *McrI* (175 + 25 bp fragment)

Quality Control

Each lot of Pwo DNA polymerase is assayed for activity on activated DNA. Furthermore a function test for PCR is performed using λDNA as well as human genomic DNA. Proofreading activity is assayed according to Fig. 1.

Each lot of Pwo DNA polymerase is assayed for contaminating activities as stated below.
See data label for lot specific values.

Absence of endonucleases

1 µg λDNA is incubated with Pwo DNA polymerase and 200 µM dNTPs each in 50 µl PCR buffer with paraffin-oil overlay at 37° C for 16 h and at 65° C for 4 h. The amount of enzyme units which showed no degradation of λDNA is stated under "Endo1".

Absence of endonuclease

1 µg λDNA *Eco* RI/*Hin*d-III fragments is incubated with Pwo DNA polymerase and 200 µM dNTPs each in 50 µl PCR buffer with paraffin-oil overlay at 37° C for 16 h and at 65° C for 4 h. The number of enzyme units which showed no alteration of the banding pattern is stated under "Endo2".

Absence of " nicking activity"

1 µg supercoiled pBR322 DNA is incubated with Pwo DNA polymerase with 200 µM dNTPs each in 50 µl PCR buffer with paraffin-oil overlay at 37°C and 65° C for 4 h. The number of enzyme units showing no relaxation of supercoiled DNA is stated under "Nick-Act.".

Absence of priming activity

100 ng of template DNA are incubated without primers with 20 units Pwo DNA polymerase in 100 µl PCR buffer with paraffin-oil overlay. As analyzed by agarose gel electrophoresis, no DNA synthesis occurs.

References

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6 Cha, R. S. et al. (1992) *PCR Methods Applic.* **2,** 14–20.
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[1] Nonidet is a trademark of Rohm & Haas, Philadelphia, USA.
[2] Tween is a trademark of ICI Americas Inc., Wilmington, USA.
[3] AmpliWax is a trademark of The Perkin-Elmer Corporation, Norwalk, USA.
[4] Triton is a trademark of Rohm & Haas, Philadelphia, USA.
Pwo DNA Polymerase is covered by U.S. patent 5.352.778 and 5.500.363.

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